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# Biodiversity Study of Intracellular Bacteria Closely Associated with Paralytic Shellfish Poisoning Dinoflagellates *Alexandrium tamarense* and *A. minutum*

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***Abstract***

Paralytic shellfish poisoning (PSP) toxins are potent environmental neurotoxins mainly produced by marien dinoflagellates. *Alexandrium* spp. are the most abundant and widespread producer of saxitoxin (STX). Currently, the evolutionary history that resulted in the curious cross-kingdom distribution of STX-synthesis remained unclear. However, the increasing number of findings of toxin-producing bacteria in dinoflagellate supports the hypothesis of the bacterial origin of PSP toxins. Addtionally, evidence that there are specific symbiosis bacterial taxa associated with phytoplankton indicates the presence of specific selective mechanisms between them, and implies that the symbiosis bacteria have some vital functions to the benefit of the dinoflagellates. Culture-independent high-throughput pyrosequencing analysis is able to provide a thorough description of microbiota community in environmental samples, which also helps to reveal their potential function. This paper aims to demonstrate the biodiversity of the bacteria closely associated with two toxic dinoflagellate strains, *A. tamarense* and *A. minutum* using the pyrosequencing method.

***Keywords***

*Toxic Dinoflagellate; Alexandrium Tamarense ; Alexandrium Minutum; Paralytic Shellfish Poisoning; Intracellular Bacteria*

## Introduction

Paralytic shellfish poisoning (PSP) toxins including saxitoxin (STX) and its 58 analogues based on a tetrahydropurine skeleton and varied in their toxicity are potent environmental neurotoxins (Steidinger KA et al 1993; Thottumkara AP et al 2014; Zhang F et al 2013). Annually, PSP toxins cause estimated global loss of milliard dollars and death of over 300 peoples in the world (Etheridge SM et al 2010; Green DH et al 2004). Marine dinoflagellates such as *Alexandrium* spp., *Pyrodinum bahamense* var. *compressum* and *Gymnodinium catenatum* are the main PSP producers (Gallacher S et al 1997; Steidinger KA et al 1993; Zhang F et al 2013). In particular, *Alexandrium* spp. is the most abundant and widespread producer of STX (Thottumkara AP et al 2014; Zhang F et al 2013). Eight of the 30 known species within the genus can produce STX (Orr RJ et al 2013; Thottumkara AP et al 2014). Additionly and interestingly, some bacteria isolated from the toxin-producing dinoflagellates are also found to produce PSP toxins (Orr RJ et al 2013; Simidu U et al, 1990; Zhang F et al 2013). These findings provide more supporting evidence for the hypothesis of a bacterial origin of PST. This hypothesis has been investigated by numerous studies, though the results were conflicting (Kodama M et al 1990; 1996; Silva ES 1979). There is also increasing evidence that there are specific symbiosis bacterial taxa associated with phytoplankton, indicating the presence of specific selective mechanisms, and implying that the symbiosis bacteria have some functions to the benefit of the alga, and these interactions could be the product of co-evolution between bacteria and algae over millions of years (Dantzer WR et al, 1997; Green DH et al. 2004; Jasti S et al. 2005).

The evolutionary history that resulted in the curious cross-kingdom distribution of STX-synthesis remained unclear. Increasing number of studies have reported the direct observation of intracellular bacteria in toxic species of dinoflagellates (Dantzer WR et al, 1997; Gallacher S et al 1999; Green DH et al. 2004; Jasti S et al. 2005). Although toxigenic bacteria could be isolated from toxic dinoflagellates, it was not clearly proven whether the isolated

bacterial strains and the corresponding intracellular bacteria were the same because of marine microbial cultivability (Gallacher S et al 1999; Jasti S et al. 2005; Zhang F et al 2013). Currently culture-independent high-throughput pyrosequencing analysis is able to provide a thorough description of microbiota community in environmental samples, which also helps to reveal their potential function (McCann JC et al 2014, Zhang XL et al 2015). This paper is aimed to demonstrate the biodiversity of the intracellular bacteria closely associated with toxic PSP-producing dinoflagellate, *Alexandrium tamarense* and *Alexandrium minutum*, using pyrosequencing method.

## Experimental Section

The two dinoflagellate strains, *Alexandrium tamarense* (880#) and *Alexandrium minutum* (amtk-3) were kindly provided by Prof. Hung-Nong Chou at National Taiwan University. An axenic cultures of both strains were cultured in f/2 medium. Cultures were kept at 25°C and a 12 h light:12 h dark cycle. LC-MS-MS analysis of the STX toxin production of the isolated microbial strain was performed according to the method reoprted previously (LC-MS-MS analysis of the STX toxin production of the isolated microbial strain was performed according to the method reoprted previously (Steidinger KA et al 2013, Zhang XL et al 2011). Molecular analysis of the sxtA biosynthesisi gene was performed according to the method reoprted previously (Steidinger KA et al 1993). With the light intensity of ca. 200 μmol photons $m^{-2}$•$S^{-1}$. Genomic DNA of the samples was extracted using Wizard® DNA Kit (Promega, Madison, USA) following the manufacturer's instruction. The quality of extracted DNA was checked by 0.8% agarose gel electrophoresis and spectrophotometry (optical density at 260 nm/280 nm ratio). The V3-V4 region of bacterial 16S rRNA was amplified by PCR for high-throughput pyrosequencing. The 16S rRNA gene V3-V4 region of bacteria was amplified using the universal primers of the forward 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse 806R (5′-GGACTACHVGGGTTCTAAT-3′). PCR amplifications of the 16S rRNA V3-V4 region were performed. The amplicon mixture was applied to the HiSeq 2500 MiSeq Genome Sequencer (Illumina, San Diego, CA, USA). Alignment of the extracted high-quality sequences were performed using PyNAST and UCLUST. The unique sequence set was classified into operational taxonomic units (OTUs) under the threshold of 97% identity using UCLUST. Chimera Slayer was applied to remove the potential chimeric sequences in the representative set of OTUs. MOTHUR software was used for data analysis.

## Results and Discussions

As shown in Table 1, the numbers of unique and classifiable representative OTU sequences for the bacteria were 102 and 96, respectively. Addtional, the Shannon index, Simpson diversity index, Chao1 and observed species of each sample were used to evaluate the species richness and diversity indicates large distinction of species richness and diversity of the bacterial community within the sample. Based on homologous sequence alignment method and clustering with information extracted from the RDP and BLAST databases, the lowest level of taxonomy of the identified OTUs was determined. As shown in Fig. 1, the Shannon diversity curves for the two samples reached the saturation phase, indicating the majority of bacterial phylotypes of the sample had already been covered.

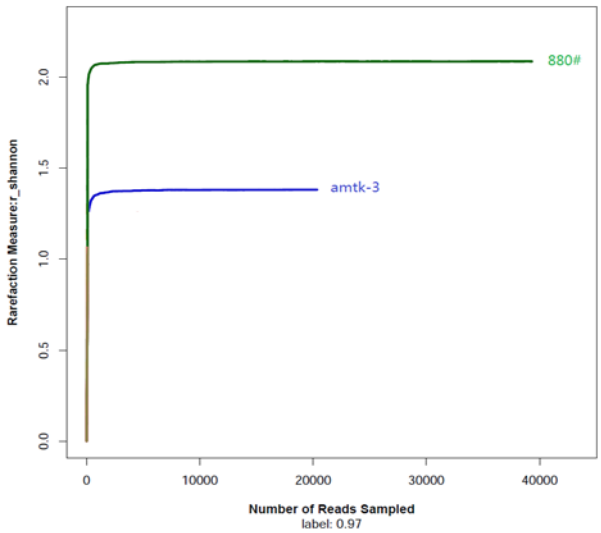



FIG. 1 THE SHANNON DIVERSITY CURVES OF THE TWO SAMPLES

TABLE 1 STATISTICS DATA OF THE OBTAINED SEQUENCES

| Sample no. | OTU | coverage | Ace index | Chao1 index | Shannon index | Simpson index |
|---|---|---|---|---|---|---|
| **880#** | 102 | 0.999845 | 101 | 103 | 2.12 | 0.09 |
| **amtk-3** | 96 | 0.999537 | 98 | 105 | 1.42 | 0.08 |

Five main bacterial genus among total 26 known were identified in the strain 880# of *Alexander tamarense* culture in this study (Fig. 2), namely *Lactococcus*, *Porphyrobacter*, *Solibacillus*, *Dinoroseobacter* and *Roseovarius.* The two predominant genus were *Lactococcus* and *Porphyrobacter*, accounting for 23.78% and 21.47%, respectivly, of the total bacterial sequences. For strain amtk-3 of *Alexander minutum*, only two main bacterial genus among total 9 known, *Microcystis* and *Porphyrobacter* were found, and the overwhelmingly predominant genus was *Microcystis*, accounting for 76.87% of the total bacterial sequences. It clearly showed that species richness and obvious diversity difference of the bacterial community existed within the two samples studied. Based on the phylogenetic analysis, there is still 3.5～8.9% unknown data could be further assigned to the potential new species. These pyrosequencing anslysis result was in good consistent with the bacterial diversity anslysis baed on tradational culture-dependent microbial isolation.

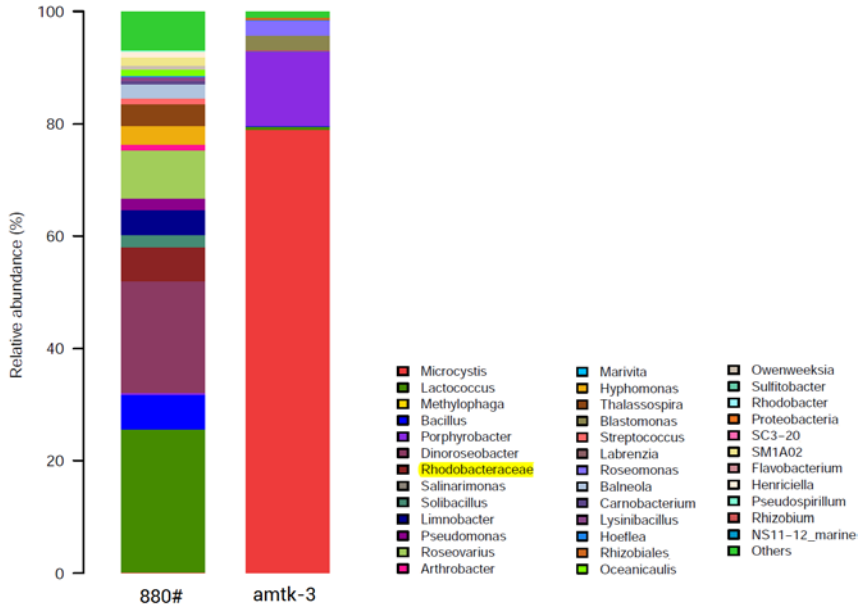
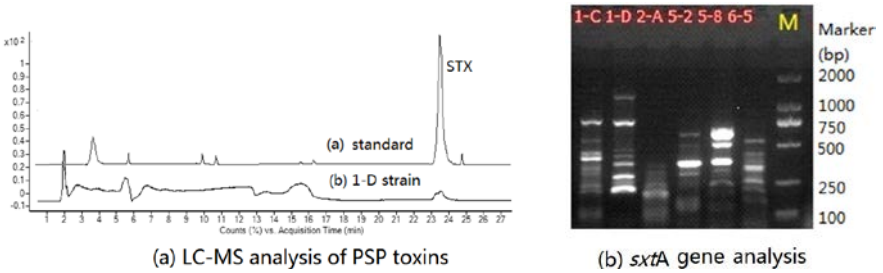



FIG. 2 MICROBIAL BIODIVERSITY ANALYSIS OF THE BACTERIA COMMUNITY FROM *A. TAMARENSE* AND *A. MINUTUM* CULTURES

It has been reported that, *Sxt*A, the unique starting gene for STX synthesis in cyanobacteria, possiblely origins from an actinobacterial specie or a Proteobacterium by independent horizontal gene transfers (HGTs) and gene fusion (Moustafa A et al 2009). In order to elucidate the relationship of the intracellular bacteria with the host, the combination study using LC-MS-MS analysis of STX production and *sxt*A biosynthesis gene analysis for some of the isolated strains for *A. tamarense* culture was performed. The result is shown in Fig 3. It indicated that, 1-D strain, one of intracellular bacteria strains isolated from the toxic *A. tamarense* produces key biosynthetic intermediate of PSP toxins, STX. In addtion, this bacteria stain has possible toxic biosynthetic gene as its gene sequence obtained in this study has high similarity with dinoflagellate *sxt*A gene. This new finding implies that the intracellular bacteria may have intracellular symbiosis relationship and potential function to the benefit of the toxic dinoflagellate.




(a) LC-MS analysis of PSP toxins (b) *sxt*A gene analysis

FIG. 3 TOXIC INTRACELLULAR BACTERIA ANALYSIS BY (A) LC-MS-MS ANALYSIS, AND (B) SXTA BIOSYNTHESIS GENE ANALYSIS

## Conclusion and Prospects

This study demonstrated the biodiversity of the bacteria closely associated with two toxic dinoflagellates, *A. tamarense* and *A. minutum*, using the pyrosequencing method. This study showed that microbial species richness and obvious diversity difference of the bacterial community existed within two dinoflagellate samples studied. It will be valuable to develop the subsequent interaction study of intracellular bacteria with the dinoflagellate. Moreover, it will also provide more scientific supporting evidence for the hypothesis of a bacterial origin of PST toxins in the near future.

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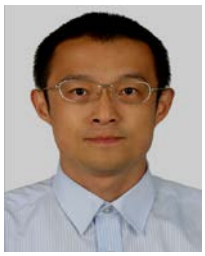

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